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Evaluation of three photosynthetic species smaller than ten microns as possible standard test organisms of ultraviolet-based ballast water treatment

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ABSTRACT

The Ballast Water Management Convention (BWMC) establishes limits for viable organisms in discharged ballast water. However, organisms smaller than 10 μm are not considered in this regulation although they represent, in some regions, the majority of the phytoplankton organisms in marine water. The objective in this study is to assess three photosynthetic species smaller than 10 μm as potential standard test organism (STO) in experimentation focused on the inactivating efficacy of ultraviolet treatments (UV). A growth modelling method was employed to determine the reduction of the viable cell concentration under either light or dark post-treatment conditions to evaluate the importance of the photoreactivation. In spite of its moderate growth rate, the high UV resistance in combination with the abundance and worldwide distribution of *Synechococcus* sp. and the environmental importance of this species constitute important reasons for considering *Synechococcus* sp. as a valuable STO for ballast water treatment.

1. Introduction

Biological invasions by non-indigenous species are one of the primary environmental problems deriving from the current globalization (Seebens et al., 2013, 2016). Ballast water is one of the main ways of spreading species that may become invasive and result in environmental, economic, and social problems at the recipient regions (Ruiz et al., 2000; Bax et al., 2003; Bailey, 2015; Gallardo et al., 2016). Depending on the sources that were consulted, the estimated annual exchanges of ballast water varies between 3 and 5 (Lloyd's Register, 2015) and 10 billion tonnes (Tsolaki and Diamadopoulos, 2010; UNCTAD, 2017). The problem of spreading species through ballast water led to the International Maritime Organization (IMO) adopting the International Convention for the Control and Management of Ships' Ballast Water and Sediments (BWMC) (IMO, 2004), which entered into force on 8 September 2017. The most recent updates as of 1 December 2020 indicate that 86 countries, representing approximately 91% of the total tonnage of the global merchant fleet, have signed the BWMC (IMO, 2020a). In addition, the United States of America has instituted their own ballast water management regulations (USCG, 2012) with some differences with respect to the IMO BWMC (Čampara et al., 2019).

The regulation D-2 (Ballast Water Performance Standard) of the BWMC establishes standards for the maximum concentration of viable organisms in discharged ballast water (Table 1), understanding viable organisms as those that are able to successfully generate new biological entities (IMO, 2018). For reaching the D-2 standards, the use of a ballast water management system is needed. According to the D-3 regulation (Approval requirements for Ballast Water Management Systems), a BMWS must be approved by the Administration (government of the state authority under which the ships are operating). The process for the approval of the ballast water management systems include land-based and shipboard tests for determining the organisms' inactivating efficacy and environmental acceptability of the BMWS; land-based testing means a test of the BWMS performed in a laboratory, equipment factory, or pilot plant (IMO, 2018). The majority of the eighty-four BWMSs approved until 3 September 2020 (IMO, 2020b), consist of a filtration or

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Table 1
IMO standards for ballast water discharge.

Organisms size	Concentration
Greater or equal to 50 μm in minimum dimension	Less than 10 viable organisms per m^3
Between 10 and 50 μm in minimum dimension	Less than 10 viable organisms per mL

Indicator microbes	Concentration
Vibrio cholerae (O1 and O139)	Less than 1 colony forming unit per 100 mL or less than 1 cfu per 1 g (wet weight) zooplankton samples
Escherichia coli	Less than 250 colony forming units per 100 mL
Intestinal enterococci	Less than 100 colony forming units per 100 mL

mechanical separation followed by a physical or chemical treatment or a combination of both (Tsolaki and Diamadopoulos, 2010; Davidson et al., 2017). The most common treatments are UV (40 BWMSs) and electrochlorination (22 BWMSs) although there are other treatments such as ultrasounds, deoxygenation, heat, or ozone, among others (Gerhard et al., 2019; Hess-Erga et al., 2019). The treatments can be applied during a ballasting and/or de-ballasting procedure or even during a voyage.

UV radiation is one of the most common disinfection treatments for effectively damaging the DNA and other cellular structures that may lead to the inhibition of reproduction or to cell death (Santos et al., 2013; Giannakis et al., 2016). Additionally, it is safe to use and environmental friendly because of the minimum production of disinfection by-products (Werschkun et al., 2012). Despite all of these benefits of UV radiation as a method for ballast water disinfection, the repair mechanisms of the DNA by the organisms (photorepair in presence of light and dark-repair with independence of light availability) after irradiation is one of the deficiencies of the application of this technology (Nebot Sanz et al., 2007; Rastogi et al., 2010). In this context, the lack of a residual effect of this treatment allows the regrowth of viable organisms, including the ones that were possibly not affected by the irradiation and those that recovered their viability by means of repair mechanisms (First and Drake, 2014; Grob and Pollet, 2016). Since UV radiation primarily affects the viability of the organisms (ability to reproduce), appropriate techniques for determining the concentration of viable organisms such as the Most Probable Number (MPN) or growth modelling are commonly used (Steinberg et al., 2011; Cullen and MacIntyre, 2016; Romero-Martínez et al., 2016). UV treatment applied during the ballasting procedure involves a dark period after the irradiation. In the Code for Approval of Ballast Water Management Systems (BMWS Code), the test water used for regrowth tests should be generated during shipboard and/or land-based test(s) and a regrowth test period of at least five days after a physical and/or chemical treatment is mandatory (IMO, 2018). The absence of light limits the photorepair process of bacteria and microalgae in ballast water, and a post-treatment dark period can increase the efficacy of the treatment (Colmenares, 2011; Moreno-Andrés et al., 2016; Romero-Martínez et al., 2020).

The BWMC also encourages the signing countries to promote and facilitate scientific and technical research on ballast water management. In this context, the BWMC currently has some points to revisit such as the typology of the organisms controlled by the D-2 regulation and the methods used for evaluating the efficacy of the treatments. The organisms with a smaller than 10 μm minimum dimension are not considered in the regulation D-2 of the BWMC. However, different studies in North American waters and in the North Sea showed that the majority of the phytoplankton organisms in natural marine water were smaller than this, and many of them can be toxic with the corresponding risk for the environment and human health in the case of blooms (Marschall, 1985; van der Star et al., 2011; Trindade de Castro and Veldhuis, 2019). Besides, the cyanobacteria *Prochlorococcus* and *Synechococcus*, both smaller than 10 μm, are worldwide distributed with high abundance (Flombaum et al., 2013). Another possible problem of the smaller sized organisms is their commonly high growth rate that favours the recovery of the population after the treatments from a small concentration of organisms (Kagami and Urabe, 2001). For evaluating the inactivating efficacy of the treatments, test organisms are commonly used. Some of their ideal characteristics are the ease of culturing and assaying, the ubiquitous presence in the marine environment, a high growth rate and, in the case of UV treatments, a relative resistant to UV exposure (Sun and Blatchley, 2017).

In this work, three photosynthetic species under a 10 μm minimum size are studied as standard test organisms (STO) for UV inactivation: *Phaeodactylum tricornutum* (oval morphotype: 8 × 3 μm), *Synechococcus* sp. (spherical with 2–3 μm of diameter), and *Anabaena* sp. (filamentous formed by numerous cells; single cell: 3,3–9,5 × 2–6,3 μm) (Lewin et al., 1958; Olenina, 2006; Prasanna et al., 2006). A high growth rate (Morais et al., 2009; Bañuelos-Hernández et al., 2015), a worldwide distribution (Martin-Jézéquel and Tesson, 2013), and its use as a test organism for water quality (ISO, 2016) make *P. tricornutum* an interesting target organism. It has three common morphotypes (oval, fusiform, and triradiate) with different sizes, however, the one selected for this study, the oval morphotype, is under the 10 μm minimum size (Lewin et al., 1958). On the other hand, the cyanobacteria *Synechococcus* sp. and *Anabaena* sp. are interesting due to their worldwide distribution as well as their ability to form blooms and produce toxins (Burja et al., 2001; Fristachi et al., 2008) with increasing frequency because of the effects of climate change (Bailey and Grossman, 2008; O'Neil et al., 2012; Lürling et al., 2013). *Synechococcus* represents one of the two major genera with high abundance in numerous oceanic regions (Partensky et al., 1999; Zwirglmaier et al., 2008; Flombaum et al., 2013). Some strains of *Anabaena* are capable of adapting to different salinities and moving between habitats with different salinities or with fluctuating salinities (Stulp and Stamp, 1984).

The objective of the present paper is to quantify, model, and compare the impact of UV-C doses combined with or without a subsequent dark period on three species smaller than 10 μm in minimum size (*P. tricornutum*, *Synechococcus* sp., and *Anabaena* sp.) to assess their suitability as STO in this size range for ballast water disinfection experimentation.

2. Material and methods

2.1. Organisms, culture medium, and pre-treatment procedure

The target organisms were the diatom *P. tricornutum* oval morphotype (CCMM 07/0402), the cyanobacteria *Synechococcus* sp. (CCMM 01/0201), and *Anabaena* sp. (CCMM 01/0101); they were provided by the Marine Microalgal Culture Collection of the Institute of Marine Sciences of Andalusia. The culture medium was ground saltwater from the Campus of Puerto Real at the University of Cadiz (pH = 7.65; conductivity at 20 °C = 48.9 mS cm^{-1} and salinity = 35.8) that was sterilized in an autoclave at 121 °C and subsequently enriched with Guillard f/2 medium (Guillard and Ryther, 1962). In the case of *P. tricornutum*, 500 μg L^{-1} of silicate was also added. Cultures were maintained in a culture chamber at 20 °C with a 24 h light cycle with photosynthetically active radiation of 36 μEinstein m^{-2} s^{-1} for *P. tricornutum* and *Synechococcus* sp., and 13 μEinstein m^{-2} s^{-1} for *Anabaena* sp. (QSL-2100 Radiometer, Biospherical Instruments Inc., San Diego, CA, USA). An aliquot of the stock culture was diluted into sterilized medium and grown for two days until a concentration was achieved of approximately 10^6 cells mL^{-1} for *P. tricornutum* and *Synechococcus* sp. and 2 · 10^5 cells mL^{-1} for *Anabaena* sp. and then subjected to the UV-C treatment. The purpose of this pre-treatment incubation is to allow the acclimation of the organisms and thus avoid a lag phase after the UV-C irradiation due to causes other than the treatment (MacIntyre and Cullen, 2005).

2.2. Experimental procedure

2.2.1. UV reactor

Samples were irradiated with a collimated beam reactor (CBR) that was designed and built according to the US EPA methodology (U.S. EPA, 2006), equipped with a low Hg-pressure monochromatic (254 nm) UV-C lamp of 10 W (Wedeco Rex UV systems, Madrid, Spain), and previously used in other studies of the research group (Romero-Martínez et al., 2014; Moreno-Andrés et al., 2016, 2019). The distance from the lamp to the sample surface was 20 cm. UV-C irradiance reaching the surface of the target culture was measured with a radiometer (PCE-UV36, PCE-Iberica). Mean intensity (I_m) was calculated according to the protocol of Bolton and Linden (2003), and the UV dose that was applied was determined as the product of the I_m and the exposure time.

2.2.2. UV irradiation and incubation

In each of the experimental series, aliquots of 20 mL of the target culture were placed on Petri plates and then irradiated with UV-C radiation for different amounts of time ranging from 1 to 20 min to obtain samples exposed to different UV-C doses (Fig. 1). Twenty minutes before the experiment, the UV-C lamp and the refrigeration air pump was activated. The irradiance of the lamp, the temperature of the reactor, and the temperature of the culture were measured between the different irradiations, and no changes were observed. For each experiment 500 mL of culture were prepared, in a flask, for all of the doses. Each time a new Petri plate was prepared for being exposed to the UV light, the flask with the culture was shaken to ensure homogeneous distribution. Throughout the irradiation, the target water was continuously homogenised with a magnetic stirrer. For every UV-C dose that was applied, 40 mL of treated sample (the content of 2 Petri plates irradiated sequentially) were collected into one borosilicate flask and another 40 mL were collected in another borosilicate flask that was covered with aluminium foil to avoid their exposure to environmental light after the UV-C irradiation. The same protocol but without UV-C irradiation was applied to the initial culture, obtaining a control for samples subsequently exposed to environmental light and a control for samples kept in the dark. Flasks with treated samples and controls were incubated in a culture chamber in similar conditions as those used in the pre-treatment incubation. Five days later, the aluminium foil was removed from all the covered flasks and all samples continued their incubation until continuous growth was detected. The UV irradiation experiments were conducted three times for each species.

2.2.3. Growth monitoring

Before the treatment assays, three techniques for monitoring the cell growth of the cultures were assessed: cell count with a microscope (Leica, DM 750; digital camera Leica, ICC 50 HD) and a Neubauer chamber (Blau Brand), absorbance with a spectrophotometer (Jenway 7315), and fluorescence with a Microplate Fluorescence Reader (Tecan infinite F200; software Tecan i-control, 1.6.19.2; plate Corning 96 Flat Bottom White Polystyrol). For determining the most suitable technique, the sensitivity, the volume of culture needed for analysis (in the experiments with the collimate beam reactor, the sample volume is limited), and the accuracy of the measurements were taken into account. Fluorescence was selected as the technique for monitoring the culture growth because of the good correlation with cell concentration, the high sensitivity of the measures, the small amount of culture needed for the analysis (0.9 mL for a triplicate) and the accuracy of the measurements. Background fluorescence (fluorescence signal in the absence of organisms) was not detected, and a regression analysis between fluorescence and concentration reported no significant intercept; therefore, the analysis was repeated forcing the intercept equal to zero (Fig. 2). Slopes were $3.38 \cdot 10^{-4}$ for *P. tricornutum* ($R^2 = 0.882$; $n = 31$), $6.20 \cdot 10^{-5}$ for *Synechococcus* sp. ($R^2 = 0.885$; $n = 34$) and $5.74 \cdot 10^{-4}$ for *Anabaena* sp. ($R^2 = 0.782$; $n = 20$).

2.3. Determining the concentration of viable organisms after the treatment

Growth curves were obtained by the representation of fluorescence measurements (as an approach of the cell concentration) in logarithmic scale versus time of exposure to environmental light in the culture chamber (i.e. the day 0 for samples kept in the dark for five days corresponds to the time when the aluminium foil was removed, the fifth day after the UV-C irradiation). To determine the concentration of viable organisms after the treatment that includes both the organisms that kept and those that recovered their ability to reproduce (Weber, 2005), the data obtained from the detection of consistent growth to the end of the incubation were fitted according to the Verhulst logistic model (Verhulst, 1830; Peleg et al., 2007) (Eq. (1); in which N_v: concentration of viable organisms at the time equal to t; N_{v0}: initial concentration of viable organisms; N_{max}: carrying capacity; r: growth rate). It was confirmed that the treatment did not modify the cell concentration-fluorescence in these data used to determine the N_{v0}. A complete description and assumptions of this application of the logistic model for determining the concentration of viable organisms after the treatment are disclosed in previous research (Romero-Martínez et al., 2016, 2019,

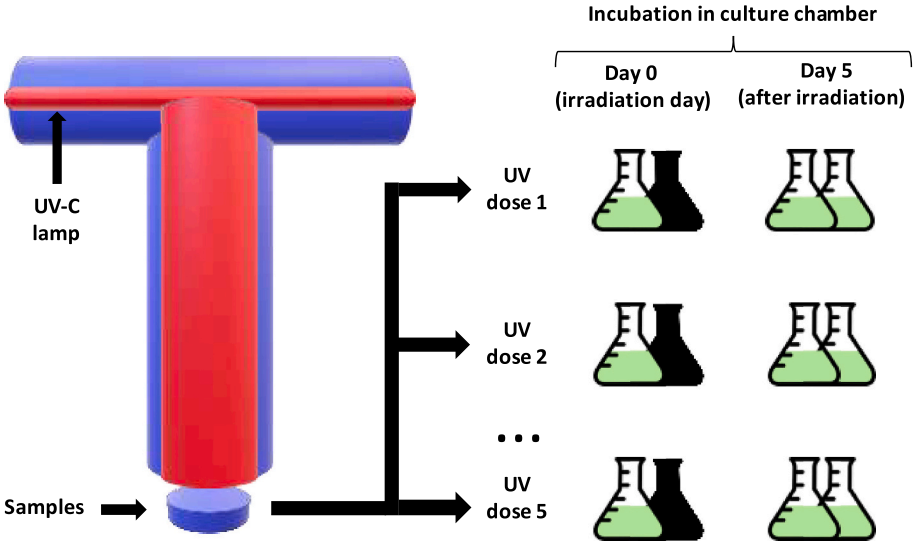

Fig. 1. Scheme of the collimated beam reactor and experimental procedure.

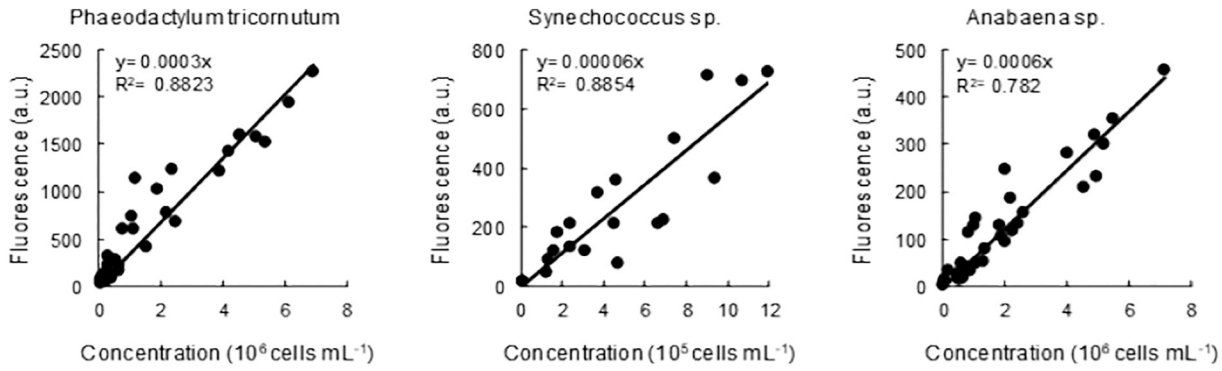

Fig. 2. Linear relation between cell concentration measured by microscopy - Neubauer chamber and fluorescence. Fluorescence measurements are in arbitrary units.

2020, 2021).

$$N_v\ (t) = \frac{N_{v0} \cdot N_{max} \cdot e^{rt}}{N_{max} - N_{v0} + N_{v0} \cdot e^{rt}} \quad (1)$$

For every sample, the Solver tool of MS Excel was applied to determine the values of r, N_{max}, and N_0 that minimize the mean quadratic error between estimated and experimental values. The values of N_0 were used for ascertaining the survival (S) as the quotient between N_0 determined in treated samples and N_0 determined for the control of the same experimental series that was exposed to environmental light in the culture chamber immediately after the irradiation. This implies that the values of S include the effect by the UV-C irradiation in samples exposed directly to environmental light and the effect of both the UV-C irradiation and the dark hold in samples kept in the dark for five days. Inactivation curves were determined for every species and illumination conditions after the UV-C treatment by representing the values of S against the UV-C dose that was applied.

The GInaFiT tool for MS Excel (Geeraerd et al., 2005) was used for fitting the inactivation curves to the best inactivation model and obtaining the corresponding inactivation kinetics parameters. These parameters were compared for evaluating the different sensitivities of the three species to the UV-C irradiation and the impact of the illumination conditions after the irradiation on the inactivating efficacy of the treatment. The UV dose necessary to achieve one log reduction (D_1) was calculated as a parameter that integrates the effect of the UV-C radiation and the darkness, allowing to directly compare the efficacy of the treatment. The Statgraphics Centurion v.16 tool was used for determining the statistical significance of the effect by the dark post-treatment and by the combination of it with the UV-C radiation on cell viability.

3. Results

3.1. Modelling of the growth curves

Growth curves were obtained by representing the logarithm of the

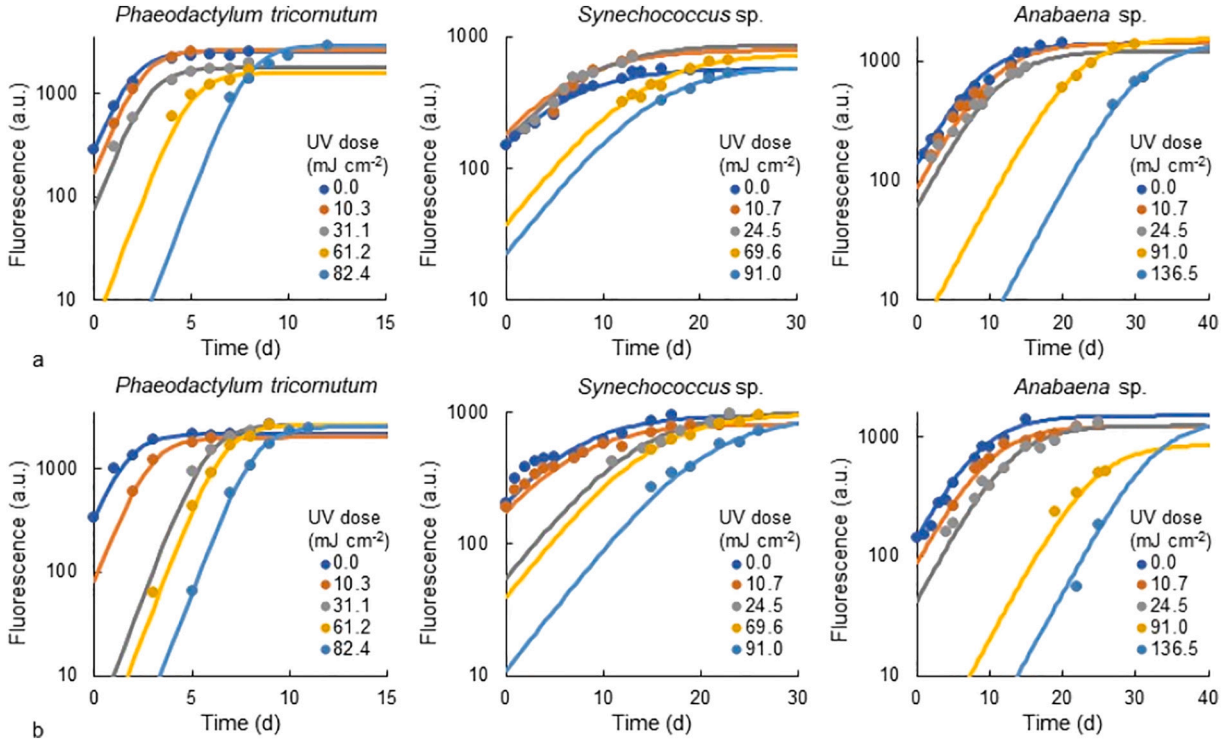

Fig. 3. Growth curves of one of the three experimental series performed for each treated organism. a) Growth curves of the species immediately exposed to light after UV-C irradiation. b) Growth curves with a dark post-treatment of five days after UV-C irradiation. For each sample, fluorescence data (in arbitrary units) are shown since there is a detection of continuous growth.

fluorescence versus the incubation time under ambient light (Fig. 3). Non-irradiated samples describe a logistic curve with exponential growth and followed by a deceleration on the growth as the concentration becomes more similar to the carrying capacity. Cell concentration in irradiated samples describe different behaviours at the beginning of the light incubation; some samples show slight or null growth as well as it may decrease in some samples. This phase is followed by a logistic curve as had occurred in non-irradiated samples. The logistic growth phase in irradiated samples is reached after a delay that is proportional to the UV dose that is applied. For each treated organism, the value of r was determined as the mean obtained in the modelling of the control samples (non-irradiated samples with and without dark post-treatment) (Table 2). Once the logistic phase in irradiated samples is reached, the growth curves of one determined organism become almost parallel, indicating that the growth rate (r) is independent from the applied UV dose. The relationship between the values of N_{max} and the applied UV dose was not observed. Since it is known that photorepair occurs in the first hours after radiation (Hull et al., 2017) and growth delay due to UV radiation has not been observed in other studies (Macintyre et al., 2018), the differences between the samples exposed to different UV doses are attributed to a decrease of the initial concentration of viable organisms (N_{v0}).

3.2. Inactivation curves and kinetic parameters

The inactivation curves were obtained by confronting the survival (S) against the UV doses. S exhibited a classic log-linear inactivation (Fig. 4) (Eq. (2); in which S: survival at UV dose equal to D; S_0: survival at UV dose equal to 0; k: inactivation rate; D: UV dose) (Chick, 1908). The survival was calculated as the logarithm of the quotient between the N_{v0} of the treated samples and the N_{v0} of the control. According to the k values (Table 3), the most resistant organism to UV-C was *Synechococcus* sp., followed by *Anabaena* sp. and, finally, *P. tricornutum*. The values of S_0, in samples subjected to dark post-treatment indicated an inactivating effect due to the dark exposure independently from the UV irradiation. The statistical analysis for comparing the intercept and slope from linear regression curves (further ANOVA for variables in the order fitted) demonstrated a significant reduction of the viable organisms due to the dark period (in absence of UV irradiation) for every tested organism (Table 3). The combination of UV irradiation and dark post-treatment increases the values of k. Since both samples, with and without the dark period, were subjected to the same UV doses, the difference in k is attributed to the inhibition of the photorepair in the case of samples with dark post-treatment. The statistical comparison of slopes indicated significant increasing of k by a factor of 1.8 in the case of *P. tricornutum* whereas, in the case of cyanobacteria, *Synechococcus* sp., and *Anabaena* sp., the increase was 1.5 and 1.2, respectively, however, the differences between slopes were not significant.

$$S\,(D) = S_0\, e^{-k\,D} \tag{2}$$

4. Discussion

It was not difficult to culture the three species used in this study in a general media (f/2 in our experiment) with the peculiarity in the case of *P. tricornutum* of being a diatom without silicate demand, although the presence of silica enhances the growth (Zhao et al., 2014). The growth rate of 1.15 d^{-1} in *P. tricornutum* was clearly higher than the values from other species with growth rates of 0.22 d^{-1} for *Synechococcus* sp., 0.26 d^{-1} for *Anabaena* sp., (Table 2). Additionally, r of *P. tricornutum* was higher than the value measured in other phytoplankters such as *Tetraselmis suecica* with 0.73 d^{-1} (Romero-Martínez et al., 2020), *Nannochloropsis salina* with 0.75 d^{-1} or *Chlorella marina* with 0.53 d^{-1} (Arumugam et al., 2012). Conversely, the values of r in both cyanobacteria were lower which is in concordance with results in literature (Lürling et al., 2013). Therefore, *P. tricornutum* would be a more appropriate STO because the high growth rate of this microalgae allows the monitoring of the growth curves with a shorter incubation time.

Table 2
Growth rate r and carrying capacity N_{max} of the three species (mean ± standard deviation; $n = 6$).

	r (d^{-1})	N_{max} (cell mL^{-1})
P. tricornutum	1.15 ± 0.17	$8.5 \cdot 10^6 \pm 0.7 \cdot 10^6$
Synechococcus sp.	0.22 ± 0.07	$1.3 \cdot 10^7 \pm 0.3 \cdot 10^7$
Anabaena sp.	0.26 ± 0.02	$2.1 \cdot 10^6 \pm 0.4 \cdot 10^6$

The resistance by a determined organism against the UV radiation is quantified by the inactivation rate (k): high k indicates high UV-sensitivity and thus low UV-resistance. However, the value of k determined for samples exposed to environmental light upon the UV irradiation is dependent on both the UV resistance and the photorepair ability. In this context, the comparison of the k calculated with and without dark post-treatment allows determining the importance of both features. The ideal characteristics for the STO to evaluate the UV-based treatment efficacy would be low k in both light and dark post-treatment conditions; this implies that more sensitive organisms (high k) would be inactivated with lower UV doses with respect to the STO (low k). Additionally, absence of inactivation due to the dark hold period ($S_0 = 0$) is considered a desirable characteristic because it does not introduce biases, in the modelling of the inactivation curves by loss of viability due to the dark exposure.

The resistance to UV irradiation of *Synechococcus* sp. in the samples without a dark post-treatment was 2.84 and 2.21 times higher than *P. tricornutum* and *Anabaena* sp., respectively. In the case of the samples with a dark post-treatment, the resistance of *Synechococcus* sp. was 3. 38 times higher than *P. tricornutum* and 1.76 times higher than *Anabaena* sp. (Table 3). Other studies also used radiation with UV-C lamps and analysed viability taking into account the photoreactivation effect. In these studies, k values were between 0.010 and 0.079 $cm^2\,mJ^{-1}$ for *Tetraselmis suecica* (Liu et al., 2016; Sun and Blatchley, 2017; Lundgreen et al., 2019; Romero-Martínez et al., 2020, 2021) and 0.025 $cm^2\,mJ^{-1}$ for *Tisochrysis lutea* (Romero-Martínez et al., 2016). *T. suecica* is a representative organism of the ≥10 to <50 μm size class of the D-2 discharge standard (D'Agostino et al., 2015; Olsen et al., 2016) and *T. lutea* (round shaped: 3–7.5 μm in diameter) belongs to the fraction of organisms >10 μm that are not currently included in the regulations (Heimann and Huerlimann, 2015). On the other hand, focusing on the k values in experiments with UV-C lamps and analysing the viability but with samples subjected to a dark post-treatment of five days (as our experiment), k values were between 0.095 and 0.165 $cm^2\,mJ^{-1}$ for *T. suecica* and 0.114 $cm^2\,mJ^{-1}$ for *T. lutea* (Romero-Martínez et al., 2016, 2020, 2021). The high sensitivity of *P. tricornutum* to the UV radiation and the resistance of *Synechococcus* sp. are consistent with other studies (Gao et al., 2009). Therefore, the cyanobacteria *Synechococcus* sp. has a high resistance to UV irradiation in both light and dark post-treatment conditions which supposes a desirable characteristic for being considered as a STO for evaluating the efficacy of UV treatment.

According to the differences between the values of k for either light or dark post-treatment, the three species that were studied showed different levels of photoreactivation. For both cyanobacteria, *Synechococcus* sp. and *Anabaena* sp., although k was higher in the case of dark post-treatment, these differences were not significant as reported by the statistical analysis for comparing regression curves. On the contrary, other cyanobacteria such as *Microcystis aeruginosa* showed noticeable photoreactivation (Sakai et al., 2011). In the case of the diatom *P. tricornutum*, the comparison of the regression curves reported significant differences between the samples subjected to light or dark post-treatment. The k increased from 0.054 to 0.098 $cm^2\,mJ^{-1}$ (factor of 1.8), which is not as important as for other species such as *T. suecica* for which the k values increased from 0.026 to 0.133 $cm^2\,mJ^{-1}$ (factor of 5.1) or from 0.010 to 0.095 $cm^2\,mJ^{-1}$ (factor of 9.5) (Romero-Martínez

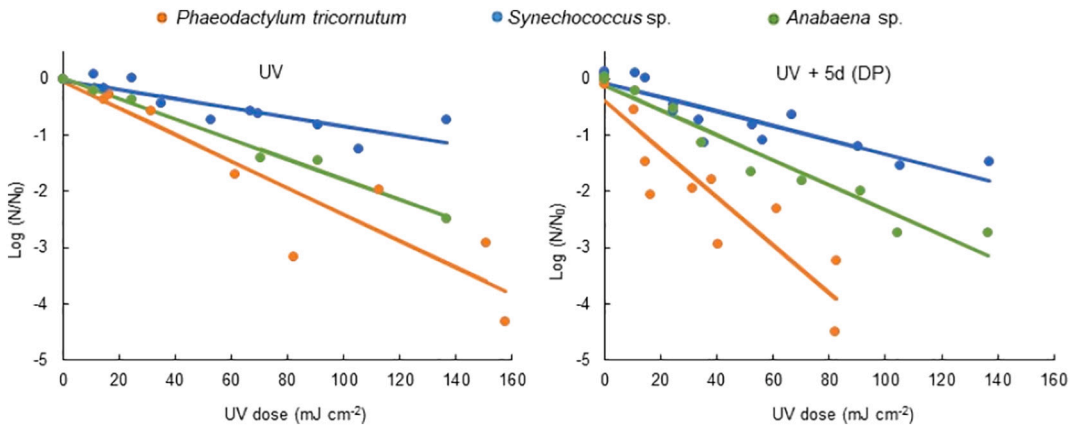

Fig. 4. Inactivation curves for the three species representing the survival versus UV doses. Survival is calculated as the logarithm of the quotient between the N_{v0} of the treated samples and the N_{v0} of the control. DP: dark post-treatment.

Table 3
DP: dark post-treatment (days). *k*: inactivation rate. Log (S_0): inactivation reached only with the dark post-treatment, indicated in orders of magnitude.

Organism	DP (d)	*k* (cm^2 mJ^{-1})	Log (S_0)	R^2	n
P. tricornutum	0	0.054 ± 0.008	−0.049 ± 0.284	0.861	10
	5	0.098 ± 0.014	−0.398 ± 0.260	0.831	12
Synechococcus sp.	0	0.019 ± 0.004	−0.018 ± 0.119	0.717	11
	5	0.029 ± 0.004	−0.063 ± 0.107	0.791	15
Anabaena sp.	0	0.042 ± 0.002	0.016 ± 0.079	0.986	6
	5	0.051 ± 0.004	−0.117 ± 0.132	0.943	10

et al., 2020, 2021) or *T. lutea* where *k* increased from 0.025 to 0.114 cm^2 mJ^{-1} (factor of 4.6) (Romero-Martínez et al., 2016). Practically all organisms present photoreactivation to a greater or lesser extent. Therefore, treating the ballast water with UV irradiation during the ballasting procedure would be more effective because the subsequent storage in the ballast tanks would increase the inactivation by avoiding the photoreactivation.

Various organisms have different sensitivity levels to prolonged dark periods by means of strategies of adaptation such as decreasing their metabolism or accumulating nutrients (Hori et al., 1982; Jochem, 1999). According to the values of S_0 (Table 3), the dark hold of five days caused the inactivation of the 60.0%, 23.6% and 13.5%, respectively, for *P. tricornutum*, *Anabaena* sp. and *Synechococcus* sp. A possible adaption to darkness and, therefore, the minimal damage produced by the five days of darkness has been previously observed in some species of the genus *Synechococcus* and the genus *Anabaena* as well as other cyanobacteria (Mannan and Pakrasi, 1993; Hood et al., 2016).

Since there are two parameters (*k* and S_0) involved in the modelling of the inactivation, the effect by the treatment can be summarized as the dose required for obtaining "n" log reductions (Hijnen et al., 2006; Macintyre et al., 2018). Within the range of the UV doses used in the experimentation, the only D_n achieved by the three species was D_1. This parameter also allows the comparison between organisms that follow different inactivation kinetic models. In all cases, the combination of the UV-C radiation with the dark post-treatment reduced the D_1 (Fig. 4), increasing the efficacy of the treatment by a 65.1% for *P. tricornutum*, 37.5% for *Synechococcus* sp. and 28.4% for *Anabaena* sp. That is, the photoreactivation was more relevant in the case of the diatom *P. tricornutum* in comparison with the two studied cyanobacteria; however other phytoplankters such as *T. suecica* and *T. lutea* with an increase of the efficacy of 81% and 92.21% respectively, showed higher levels of photoreactivation (Romero-Martínez et al., 2016, 2020) Therefore, the cyanobacteria *Synechococcus* sp. was considered an appropriate STO for evaluating the efficacy of UV treatments in terms of resistance to dark periods, resistance against UV-C radiation, and photoreactivation features. Other environmental features of *Synechococcus* sp. for considering it a good STO are its ability to dominate the aquatic environment, create blooms, produce toxins and even float in bioaerosols.

The way of irradiating the cultures in this experiment is different than the one from the BWMS where the water flows directly around the lamps. The collimate beam reactor has been selected as the UV radiation source because of the ease of controlling all of the parameters, therefore, the calculation of the UV doses is highly accurate (Blatchley, 1997). Although the same doses should produce the same inactivation effect independently of the ultraviolet source, more research should be done for confirming it and the escalation to a continuous-flow system would be needed.

5. Conclusions

Since most marine plankton, in some regions, is less than 10 μm in size, it is interesting to have some STO species of that size to evaluate the effectiveness of the treatments applied to ballast water.

The objective in this study was to assess the suitability of three photosynthetic species smaller than 10 μm as STO organisms for evaluating the inactivating efficacy of UV-based ballast water treatment.

The determination of the inactivation rate (*k*) in treated samples is essential for a better understanding of the UV irradiation effect and for a correct comparison of the resistance to it. Suitable standard test organisms (STO) would need to have a high resistance to UV irradiation that would be reflected in low *k* values. In the samples with a dark post-treatment of five days, the *k* values allow calculating the effect of the darkness on preventing the photoreactivation.

The growth modelling of the irradiated culture is an adequate method for assessing the efficacy of the treatment, therefore, a high growth rate is a characteristic that is desirable since the determination of the concentration of viable organisms after the treatment requires a shorter incubation time.

The diatom *P. tricornutum* reported the highest growth rate among the studied species. This characteristic would make *P. tricornutum* a good STO organism for inactivation experiments with other treatments that are different than UV irradiation because of its high sensitivity to UV light. Another reason for not considering *P. tricornutum* as a good STO for UV based treatments is the high photoreactivation that was observed. This makes it possible to properly treat the water, however, it would not be in compliance with the ballast water discharge standards if cell reactivation has been carried out.

Anabaena sp. demonstrates a moderate growth rate, a relatively high sensitivity to UV irradiation, and the worst Neubauer-fluorescence correlation (due to its association to filaments). These are negative reasons to advise against considering it as a good STO for marine water disinfection.

On the other hand, the cyanobacteria *Synechococcus* sp. showed the highest UV resistance, with low influence by the dark post-treatment on its survival. This implies that the UV irradiation that causes inactivation of this organism is likely to be able to inactivate less resistant organisms. The high resistance of *Synechococcus* sp. was reflected in the low UV inactivation rate (k), and no significant photoreactivation was observed. This absence of significant photoreactivation would ensure similar inactivation results by treating water during the ballasting or de-ballasting procedure. In spite of its moderate growth rate, its high UV resistance, abundance and worldwide distribution of *Synechococcus* sp., and the environmental importance of this species constitute important reasons for considering it as a valuable STO for ballast water treatment, especially if the <10 μm size class was included in the regulations.

CRediT authorship contribution statement

Ignacio Rivas-Zaballos: Conceptualization, Methodology, Formal analysis, Investigation, Writing – original draft, Visualization, Writing – review & editing. **Leonardo Romero-Martínez:** Investigation, Validation, Writing – review & editing. **Ignacio Moreno-Garrido:** Methodology, Resources, Writing – review & editing. **Asunción Acevedo-Merino:** Conceptualization, Methodology, Formal analysis, Resources, Writing – review & editing, Supervision, Project administration. **Enrique Nebot:** Conceptualization, Methodology, Formal analysis, Resources, Writing – review & editing, Supervision, Project administration, Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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